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Roscovitine differentially affects CaV2 and Kv channels by binding to the open state

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Abstract

Roscovitine potently inhibits cyclin-dependent kinases (CDK) and can independently slow the closing of neuronal (CaV2.2) calcium channels. We were interested if this drug could affect other ion channels similarly. Using whole cell recordings, we found that roscovitine specifically slows deactivation of all CaV2 channels (N, P/Q and R) by binding to the open state. This effect had a rapid onset and $EC_{50} = 54$, 120 and 54 μM for N-, P/Q-, and R-type channels, respectively. Deactivation of other channel types was not slowed, including L-type calcium channels (CaV1.2, CaV1.3), potassium channels (native, Kv4.2, Kv2.1 and Kv1.3), and native sodium channels. However, most of the channels tested were inhibited by roscovitine. The inhibition was characterized by slow development and a lower affinity ($EC_{50} = 100–300$ μM). Surprisingly, potassium channels were rapidly inhibited with an $EC_{50} = 23$ μM, which is similar to the EC_{50} for roscovitine block of cell division [Meijer, L., Borgne, A., Mulner, O., Chong, J., Blow, J., Inagaki, N., Inagaki, M., Delcros, J., Moulinoux, J., 1997. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur. J. Biochem. 243, 527–536]. Potassium current inhibition seemed to result from open channel block. The high potency of these two rapid onset effects makes them complicating factors for ongoing clinical trials and research using roscovitine. Thus, the physiology and pharmacology of slow CaV2 deactivation and potassium channel block must be explored.

Keywords: CaV2 enhancement; Kv inhibition; CaV cell lines; Bullfrog sympathetic neurons; Whole-cell patch clamp; Open channel

1. Introduction

Roscovitine is a well known membrane permeant cyclin-dependent kinase (CDK) inhibitor (Meijer, 1996; Havlicek et al., 1997), which is thought to be the mechanism by which it blocks cell division. It has been effectively used to suppress growth of over 20 different human tumor cell lines (Iseki et al., 1997, 1998; Mgbonyebi et al., 1998; Lee et al., 1999; McClue et al., 2002; Mihara et al., 2002), which led to its testing in clinical trials as an anticancer drug (reviewed by Senderowicz, 2003; Guzi, 2004).

Roscovitine was recently shown to have an additional effect to slow the closing (deactivation) of ω-conotoxin MVIIC sensitive voltage-dependent calcium channels (Yan et al., 2002), which are N-type (CaV2.2) and P/Q-type (CaV2.1) channels (Hillyard et al., 1992). In addition, Yan et al. (2002) showed a small roscovitine effect on toxin insensitive channels, which suggests that either L-type or R-type channels could also be affected. We have previously demonstrated that roscovitine slows deactivation of N-type channels by selectively binding to open

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channels to reduce transition rates between two open states (Buraei et al., 2005). However, the selectivity of roscovitine for other calcium channel types remains to be investigated.

Only antagonists were previously available for the CaV2 channel family (Triggle, 2003) while specific CaV1 (L-channel) agonists and antagonists have provided invaluable tools for the elucidation of their role in physiology and pathophysiology (Bean, 1985; Nilius et al., 1985; Nowycky et al., 1985; Sturek and Hermsmeyer, 1986; Triggle, 2003; Elmslie, 2004). We show that roscovitine specifically slows deactivation of all CaV2 channels (N-, P/Q- and R-channels). Hence, this drug can act as a long-sought CaV2 small molecule drug (reviewed by Triggle, 2003). However, another effect of roscovitine was to inhibit ionic currents with potassium channels being most potently affected. We show potassium channel inhibition results from roscovitine binding to open channels. Our results help define several potential mechanisms by which roscovitine can produce its physiological effects. These mechanisms must be differentiated when attempting to determine how roscovitine affects physiological or pathophysiological changes.

2. Methods

2.1. Cells

To study native sodium and potassium currents, paravertebral sympathetic ganglia were isolated from adult bullfrogs (Rana catesbeiana) and the neurons dissociated with collagenase/dispase digestion and trituration (Kuffler and Sejnowski, 1983; Jones, 1987; Elmslie et al., 1992). The method of sacrifice was approved by the Institutional Animal Care and Use Committee. Cells were maintained in L-15 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 4 °C until use (usually 2–14 days). Kv1.3, Kv4.2 channels, as well as CaV2.3 (R-type) and CaV1.3 (L-type) with ß$_3$ and $\alpha_2\delta$, were transiently transfected in HEK 293 cells as previously described (Schofield and Ricci, 2005). Briefly, a mixture of 0.2 μg of Kv (or 1.0 μg of each α, β and $\alpha_2\delta$ for CaV channels), 0.2 μg pEGFP-N1, and 10 μl Superfect transfection reagent in 150 μl opti-MEM was preincubated for 15 min and then applied to the culture wells of a six-well tissue culture plate seeded with HEK 293T cells (5×10^4 cells/ml). After 24 h incubation the cells were plated into 35-mm culture dishes that subsequently served as the electrophysiological recording chamber. The pEGFP-N1 vector was co-transfected to identify cells suitable for voltage-clamp analysis. Cells were cultured in DMEM supplemented with 10% fetal calf serum under an atmosphere containing 10% CO_2. Kv4.2 was a gift from L.Y. Jan (University of California, San Francisco, CA), Kv1.3 was a gift from Stephen Korn (University of Connecticut, Stores, CT), CaV2.3 was a gift from Brett Adams (Utah State University, Logan, UT) and CaV1.3e, CaVß$_3$ and $\alpha_2\delta$ were gifts from Diane Lipscombe (Brown University, Providence, RI).

Stably transfected cell lines were used to record CaV1.2 (L-type), CaV2.1 (P/Q-type), CaV2.2 (N-type), and Kv2.1 currents. CaV2.2α, ß$_3$ and $\alpha_2\delta$ where stably expressed in tsA201 cells (from Diane Lipscombe) and cultured in DMEM containing the selection agents Zeocin, Blastocin, and Hygromycin B. CaV2.1, ß$_{1b}$ and $\alpha_2\delta$, and, CaV1.2, ß$_{1a}$ and $\alpha_2\delta$ were stably expressed in BHK cells and cultured in DMEM containing G418 and methotrexate. Both BHK derived cell lines were gifts from Dr. Yasuo Mori (Kyoto University, Kyoto, Japan). The Kv2.1 expressing HEK cell line was a gift from Eckhard Ficker (MetroHealth Medical Center, Cleveland, OH), and these cells were cultured in DMEM containing G418.

2.2. Electrophysiology

Cells were voltage-clamped using the whole-cell configuration of the patch clamp technique. Pipettes were pulled from Schott 8250 glass (Garner Glass, Claremont, CA) on a Sutter P-97 puller (Sutter Instruments Co., Novato, CA). Series resistance ranged from 1.3–2.5 MΩ for sympathetic neurons and 2–7 MΩ for cell lines, and was compensated at 70–90%. Currents were recorded using an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA) and digitized with a MacAdios II analog-digital converter (GW Instruments, Somerville, PA). Experiments were controlled by a Macintosh Quadra 800 computer (Apple Computer, Cupertino, CA) running S3 data acquisition software written by Dr. Stephen Ikeda (NIH, NIAAA, Bethesda, MD). Leak current was subtracted online using a P/4 protocol. All recordings were carried out at room temperature. Whole-cell currents were digitized at 50 kHz after analog filtering at 10 kHz.

2.3. Solutions

For calcium currents the internal solution contained (in mM) 120 NMG · Cl, 10 TEA · Cl, 14 Creatine · PO_4, 6 $MgCl_2$, 1 $CaCl_2$, 10 NMG · HEPES, 5 $Tris_2$ · ATP and 11 NMG_2 · EGTA; and had an osmolarity of 300 mOsm. The external solution for CaV1.2, CaV1.3 and CaV2.1 contained (in mM) 30 $BaCl_2$, 100 NMG · Cl, 10 NMG · HEPES and 15 glucose. The 30 mM $BaCl_2$ was reduced to 5 mM $BaCl_2$ for CaV2.2 and substituted with 20 mM $CaCl_2$ for CaV2.3. NMG · Cl concentration was always adjusted to maintain an osmolarity of 315 mOsm for these external solutions. For native sodium and potassium currents internal solutions contained (in mM) 83 KCl, 14 Creatine · PO_4, 6 $MgCl_2$, 2.5 NMG · HEPES, 5 $Tris_2$ · ATP, 10 NMG_2 · EGTA and 0.3 Li_2 · GTP; the osmolarity was 220 mOsm. External solutions contained in mM 115 NaCl, 2.5 KCl, 2.5 NMG · HEPES and 2 $MnCl_2$ (instead of $CaCl_2$); the osmolarity was ~240 mOsm. To suppress sodium currents, NaCl was replaced by NMG · Cl in some experiments. For expressed potassium channels internal solution contained (in mM) 140 KCl, 1 EGTA, 10 HEPES, 4 Na_2ATP, 0.1 Na_2GTP and 4 $MgCl_2$ (300 mOsm), and external solution contained (in mM) 150 NaCl, 5.5 KCl, 10 HEPES, 6 $MgCl_2$ and 15 Glucose (315 mOsm). Solutions were titrated to pH 7.2–7.3 with either NMG base, KOH or NaOH. Test solutions were applied from a gravity-fed perfusion system with seven inputs and a single output. The minimum exchange time for this system was ~2 s.

2.4. Data analysis

Data were analyzed using Igor Pro (WaveMetrics, Lake Oswego, OR) running on a Macintosh computer. Step currents were measured as the average of 10 points at the end of voltage steps. Tail currents were measured as the average of 3 points starting ~0.3 ms into the repolarizing pulse. To measure the effect of roscovitine on deactivation, tail currents were fit with a double exponential equation using fixed τ values. The τ_{fast} was fixed to the average deactivation τ of control tails, and τ_{slow} to the average deactivation τ in 300 μM roscovitine (maximal effect). The roscovitine effect to slow deactivation was calculated as the fraction of the slow component amplitude to the total tail amplitude. Activation τ (τ_A) was determined by fitting a single exponential function to the current after a 0.3 ms delay (Jones and Marks, 1989; Buraei et al., 2005). Group data were calculated as mean ± S.D. throughout the paper. Paired *t*-test was used for in-cell comparison.

2.5. Chemicals

All experiments utilized R-roscovitine (here referred to as roscovitine) from either Calbiochem (La Jolla, CA) or LC labs (Woburn, MA). Indiribin-3′-monoxime was obtained from Calbiochem. Other chemicals were obtained from Sigma (St. Louis, MO). All solutions contained DMSO to control for its presence in roscovitine solutions. For experiments using a range of roscovitine concentrations (e.g., dose-response measurements), the DMSO concentration of all solutions was set to that in the solution with the highest roscovitine concentration (i.e., 0.6%). The DMSO in the control solutions had no effect on the whole-cell calcium currents.